## REMARKS/ARGUMENTS

The Office Action has rejected claims 15 – 19 under 35 U.S.C. § 103. In light of the amendments above and the arguments below, Applicants respectfully request reconsideration.

On October 2, 2003, Applicants' attorney, Jean C. Baker, interviewed Examiner Ulrike Winkler via a telephone conference call. Applicants and their attorney thank Examiner Winkler for the courtesy of her time and for her helpful suggestions. Applicants' attorney and Examiner Winkler agreed that Applicants will rewrite claims 17 and 18 in independent form and will provide a 37 CFR § 131 Declaration to antedate Kirchmaier, et al. Applicants' attorney also discussed the Becker, et al. reference with the Examiner and suggested providing the Examiner with a more thorough discussion of Becker, et al. provided by the inventor, William M. Sugden. As described below, Applicants have complied with the Examiner's request and Applicants' attorney's suggestions.

### § 103 Rejection

The Office Action has rejected claims 15 – 19 under 35 U.S.C. § 103 as being unpatentable over Mackey, et al., Kirchmaier, et al. and Becker, et al. The Examiner notes that "the instant invention is drawn to a method of screening candidate molecules that disrupt viral looping linking factors in a cell." The Office Action cites Mackey, et al. as teaching "that multiple regions with EBNA-1 can link DNAs" but notes that "the reference teaches an *in vitro* assay." Inventor Sugden has previously supplied a Declaration asserting that the references does not utilize whole cell extracts which would have other cellular proteins present during the binding phase of the inhibitor with the nucleic acid banding site as required by the instant claims. Applicants again draw the Examiner's attention to this

Declaration and reconfirm that Mackey, et al. cannot predict the success of the present invention. The Examiner uses Kirchmaier, et al. and Becker, et al. to rectify this deficiency.

Applicant has supplied a § 131 Declaration noting that he is the "William Sugden" who is an author of Kirchmaier, et al. As Kirchmaier, et al. is published less than a year before the November 1997 priority date of the above-identified application, Applicants note that Kirchmaier, et al. cannot be considered prior art.

Becker, et al. is used as disclosing a "cell-based assay determining viral replication/inhibition in Burkett Lymphoma cells that comprise EBV . . . ." Later on in the Office Action, Becker, et al. is used for the proposition that "there is a high expectation of success in utilizing the host cell assay methods for determining the viral replication inhibition . . . ."

Applicants assert that the use of Becker, et al. in the combination is impermissible because Becker, et al. is non-analogous art to the invention in question. Although Becker, et al. and the instant application both pertain to the field of Epstein Barr Virus studies, there are significant and quantum differences between the technology described in Becker, et al. and the above-identified application.

Applicants have supplied a Declaration of Inventor William Sugden detailing the differences between Becker, et al. and the present invention. Dr. Sugden describes Becker, et al. as describing "studies in which they [Becker, et al.] induce the lytic phase of the lifecycle of Epstein Barr Virus (EBV) resident in the EB3 cell line. Induction of the lytic phase of EBV requires EBV to escape from the latent phase of its lifecycle." In paragraphs 4 and 6, Dr. Sugden describes the differences between the latent phase and lytic phase of EBV's lifecycle in terms of the use of EBNA-1 protein. No demonstration in EBV lytic phase

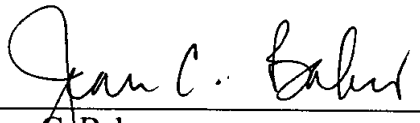
addresses any facet of the mechanism of replication of EBV DNA during the latent phase of EBV's lifecycle.

In light of the above analysis, Applicants respectfully request reexamination and allowance.

A Petition and Fee for Three Months Extension of Time and an RCE are enclosed. If further fees are necessary, please charge Deposit Account 17-0055.

Respectfully submitted,

William M. Sugden, et al.

By: 

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I hereby certify that this correspondence is being deposited with the United States Postal Services on the date set forth below as First Class Mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Date of Signature and Deposit: 1/27/04

Jean C. Baker

Jean C. Baker, Reg. No. 35,433



**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

| | | | |
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| Appl. No.: | 09/808,517 | Client Docket No.: | P01220US |
| Applicants: | William M. Sugden, et al. | | |
| Filed: | March 14, 2001 | | |
| Title: | INHIBITION OF VIRAL GENE ACTIVITIES | | |
| TC/A.U.: | 1648 | | |
| Examiner: | U. Winkler | | |
| Docket No.: | 960296.97982 | | |

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

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## DECLARATION OF WILLIAM M. SUGDEN

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Sir:

I, William M. Sugden, declare:

1. I am one of the named inventors of the above-identified application.

2. I have been asked to review the Office Action dated July 29, 2003 and respond to the Examiner's rejection. I understand that Attorney Jean C. Baker had a telephonic interview with Examiner Winkler on October 2, 2003 during which the Office Action and the Becker, et al. reference was discussed.

3. I strenuously contest the Office Action's characterization of a combination of Mackey, et al., Kirchmaier, et al. and Becker, et al. as teaching a "high expectation of success in utilizing the whole cell assay methods for determining the viral replication inhibition." I understand that the accompanying Response to Office Action will deal with issues presented by the Mackey, et al. and Kirchmaier, et al. references. I wish to discuss the Becker, et al. paper to clarify the relevancy of this disclosure to the Examiner.

4. I would like to begin by reminding the Examiner that the life-cycle of a virus comprises distinct phases. The life-cycle of Epstein-Barr virus comprises both a lytic and a latent phase. The lytic phase of the life-cycle is the reproductive cycle and results in the lysis of the host cell at the completion of viral replication. The latent phase of the growth cycle, in contrast, involves the maintenance of the viral genome within the host without host cell lysis. These two phases involve different interactions of host and viral proteins and metabolites.

5. Becker and Weinberg in their 1972 paper published in the Israel Journal of Medical Science ("Becker, et al.") describe studies in which they induce the lytic phase of the life-cycle of Epstein-Barr Virus (EBV) resident in the EB3 cell line by depriving those cells of arginine. Induction of the lytic phase of EBV requires EBV to escape from the latent phase of its life-cycle.

6. This latent phase is characterized by viral DNA replication using the viral protein, EBNA-1, in addition to much cellular machinery to occur once per cell cycle in synchrony with cell DNA synthesis. EBV's latent phase is consistent with survival and proliferation of its host cell and is the phase of the viral life cycle found in EBV-associated human cancers. Induction of the lytic phase of EBV's life-cycle leads to the amplification of viral DNA using primarily viral machinery, but not EBNA-1, expression of most viral genes, and cell death. Cells which are induced to support EBV's lytic phase cease to proliferate and die.

7. Becker, et al. treat the cells they have induced to support the lytic phase of EBV's life-cycle with distamycin A and find that this treatment inhibits the amplification of EBV DNA during the lytic phase of its life-cycle. Their studies do not address any facet of the mechanism of replication of EBV DNA during the latent phase of EBV's life-cycle. Their studies do not address any facet of EBV's life-cycle in which EBNA-1 participates.

8. The assay of the present invention will identify inhibitors of EBNA-1 required for the transcription and DNA replication mediated by EBNA-1 during the latent phase of EBV's life-cycle. Such inhibitors, for example, could be used to treat EBV-associated cancers. Such inhibitors would not score in the assay of Becker, et al. in which EBV-positive cells were induced to support the lytic phase of EBV's life-cycle and subsequently tested for inhibition of the ensuing amplification of viral DNA.

9. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

Dated: 12/19/03

William M. Sudgen, Ph.D

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Date of Signature and Deposit: 1/27/04

Jean C. Baker, Reg. No. 35,433



**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

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| Docket No.: | 960296.97982 | | |

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## § 131 DECLARATION

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Sir:

1. I, William M. Sugden, am a named inventor of the above-identified application and one of the authors of Kirchmaier, et al., 1997.

2. The first-named author, Ann L. Kirchmaier, contributed to the work described in Kirchmaier, et al., 1997 but is not properly named as an inventor in the above-identified application.

3. Kirchmaier, et al. was published in March of 1997, less than a year before the November 1997 priority date claimed by the above-identified application.

4. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the

United States Code and that such willful false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

Dated: 12/19/03

William M. Sudgen

JOURNAL OF VIROLOGY, Mar. 1997, p. 1766–1775
0022-538X/97/$04.00+0

Vol. 71, No. 3

# Dominant-Negative Inhibitors of EBNA-1 of Epstein-Barr Virus

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Received 29 August 1996/Accepted 14 November 1996

**Epstein-Barr virus (EBV) nuclear antigen 1 (EBNA-1) is required in *trans* to support replication of the EBV genome once per cell cycle via the latent origin of replication, *oriP*. EBNA-1 can also activate transcription on binding to the family of repeats of *oriP* to enhance some heterologous as well as native EBV promoters. We have made and screened derivatives of EBNA-1 for the ability to act as inhibitors of wild-type EBNA-1. These derivatives lack the linking or the retention functions of EBNA-1 and were analyzed for the residual ability to activate transcription and replication. We have identified derivatives of EBNA-1 that can inhibit up to 98% of wild-type EBNA-1's activities. We have also identified one derivative of EBNA-1 with only two of EBNA-1's three linking domains which can support transcription and replication inefficiently.**

Epstein-Barr virus (EBV) usually maintains its genome as a plasmid in cells that it infects. It contributes only one protein, EBV nuclear antigen 1 (EBNA-1), to its plasmid replication (26, 36, 48); the host cell provides all other replication machinery. EBNA-1 is a site-specific DNA-binding protein which binds to clusters of sites in EBV that together constitute the viral plasmid origin of replication, *oriP*. Purified EBNA-1 lacks detectable enzymatic functions such as a DNA-dependent ATPase or a helicase (13, 29) which are intrinsic to other viral origin-binding, replication proteins such as E1 of bovine papillomavirus type 1 or T antigen of simian virus 40. It is therefore not clear what EBNA-1 does to support replication of *oriP*-containing DNAs. EBNA-1 has additional functions; it can activate transcription from some heterologous and native EBV promoters when multiple EBNA-1 binding sites are positioned within 10 kbp of these promoters (15, 37, 45). EBNA-1 also retains DNAs to which it binds specifically in cells, as assayed by prolongation of detection of those DNAs within the proliferating cells (23, 30). However, EBNA-1's DNA binding domain (in the presence of the EBNA-1 nuclear localization signal [NLS]) is insufficient for this activity (30). EBNA-1 retains both replicating and nonreplicating DNAs in cells as measured in these assays.

EBNA-1 associates multiple DNAs in vitro, to which it binds specifically, apparently through protein-protein binding (12, 16, 27, 29, 43). When the sites bound are intramolecular, this function is termed DNA looping; when they are intermolecular or unspecified, this function is termed DNA linking. What this additional function of EBNA-1 might contribute to its support of plasmid replication is unknown. EBNA-1's ability to link DNAs to which it specifically binds (16, 27, 43) has been mapped to three regions (amino acids [aa] 54 to 89, 331 to 361, and 372 to 391) in addition to its DNA binding domain. These three regions, when fused to the DNA binding domain of Ga14, can each independently mediate linking of DNAs containing Ga14 binding sites (27).

Previous mutational studies of EBNA-1 have identified several regions of the protein that contribute to replication and activation of transcription (1, 32, 33, 47). Deletion of all but 15 amino acids of the Gly-Gly-Ala repeats, which comprise residues 90 to 328 of EBNA-1, does not affect EBNA-1's ability to activate transcription or replication (47, 48). However, deletions within the DNA binding domain of EBNA-1 that abrogate EBNA-1's ability to bind to DNA inactivate the protein, indicating that EBNA-1 must bind to DNA to activate transcription and replication. Small deletions are often tolerated throughout much of the rest of the protein, but larger deletions or combinations of small deletions generally serve to inactivate the protein (1, 32, 33, 47). We have generated derivatives of EBNA-1 (Fig. 1A) which lack one or more large domains of the protein shown to contribute to transcription, DNA retention, and DNA linking and have tested them for their functions as dominant-negative mutants. These derivatives have also been tested for the residual ability to support plasmid replication and activate transcription.

We identified derivatives of EBNA-1 that function as efficient dominant-negative mutants of EBNA-1; those that are the most efficient inhibitors of transcription and replication bind to DNA but lack the retention and linking functions of EBNA-1. We have also identified one derivative of EBNA-1 that retains two of its three linking domains, NΔ330-641, that activates transcription and replication 4 to 23% as efficiently as does wild-type EBNA-1. These findings correlate EBNA-1's replicational with its transcriptional activities.

## MATERIALS AND METHODS

**Plasmids.** The plasmids used in these experiments (Fig. 1) include the following: vector DNA encoding the hygromycin B phosphotransferase gene; effector DNA encoding the hygromycin B phosphotransferase gene and wild-type EBNA-1 (aa 1 to 641 of EBNA-1 of the B95-8 strain of EBV [28]) expressed from the cytomegalovirus (CMV) immediate-early promoter; effector DNA encoding the hygromycin B phosphotransferase gene and a derivative of EBNA-1 expressed from the CMV immediate-early promoter containing the untranslated region of exon 1 of the CMV immediate-early gene, i.e., NΔ450-641 (aa 379 to 386 and 451 to 641 of EBNA-1 [30]), NΔ330-641 (aa 331 to 641 of EBNA-1), NΔ450-618, (aa 379 to 386 and 451 to 618 of EBNA-1), or NΔ450-458,483-641 (aa 379 to 386 and 451 to 458 fused in frame to aa 483 to 641 of EBNA-1); reporter plasmids FR-TK-Luc (29), *oriP-Bam*HI C-Luc (wild-type aminoglycoside phosphotransferase II gene, derived from *oriP-Bam*HI C-CAT [45]), and DS-*Bam*HI C-Luc (wild-type aminoglycoside phosphotransferase II gene, generated by substituting yeast DNA for the family of repeats [FR] between *Sph*I and *Mlu*I in *oriP-Bam*HI C-Luc); and control plasmids for replication assays (competitor [no *oriP*, 220-bp deletion between the *BsaA*I and *Msc*I sites within the aminoglycoside phosphotransferase II gene] and *oriP*-minus [no *oriP*, 232-bp insertion of ϕX DNA at the *Msc*I site within the aminoglycoside phosphotransferase II gene]).

**Cell lines.** The cell lines used for the transient transcription assays include DG75, an EBV-negative Burkitt's lymphoma cell line (4), and 143B, an EBV-

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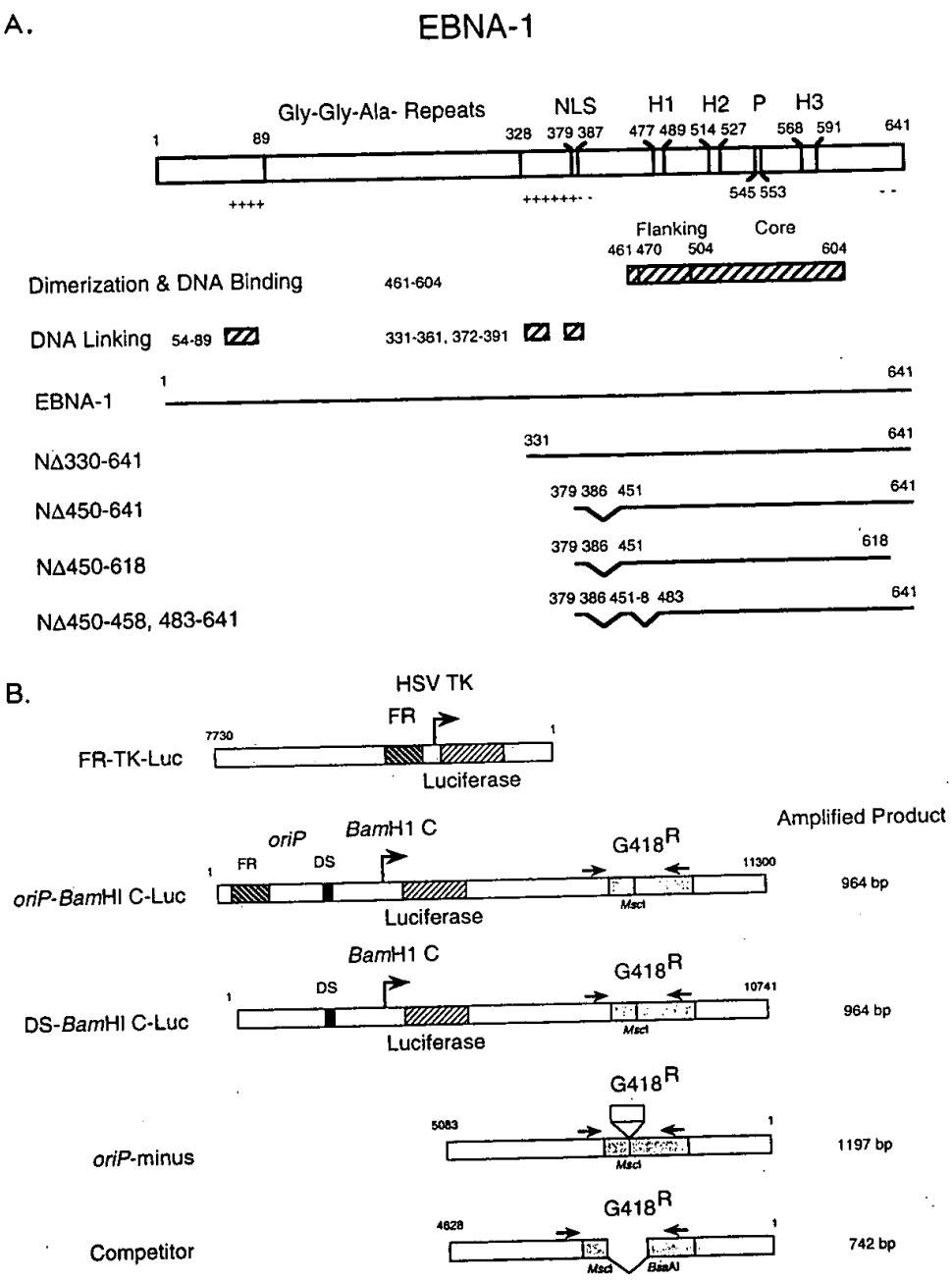


FIG. 1. Derivatives of EBNA-1 and reporter constructs. (A) Schematic representation of wild-type EBNA-1 and derivatives of EBNA-1. The Gly-Gly-Ala repeats, the EBNA-1 NLS, the linking domains (16, 27), and the DNA binding and dimerization domain of wild-type EBNA-1 (5) are indicated. The three helices (H1, H2, and H3) and the proline-rich loop (P) within the flanking and core region of the DNA binding and dimerization domain are noted (3, 5). Derivatives of EBNA-1, NΔ330-641, NΔ450-641 (30), NΔ450-618, and NΔ450-458,483-641, contain the EBNA-1 NLS (aa 379 to 386) at the amino terminus. The nomenclature indicates that the N terminus of EBNA-1 has been deleted through the amino acids noted in these derivatives, and the amino acids of EBNA-1 present in addition to the NLS are indicated. For example, NΔ450-641 contains the NLS of EBNA-1 and aa 451 to 641 of EBNA-1. (B) Maps of reporter constructs for transcription and replication assays and control constructs used for the quantitative PCR assay. FR-TK-Luc contains the FR immediately upstream of the HSV type 1 TK promoter driving luciferase (29). *oriP-Bam*HI C-Luc contains *oriP* in its natural context in EBV DNA relative to the *Bam*HI C promoter driving luciferase and contains the wild-type aminoglycoside phosphotransferase II gene (10) encoding drug resistance to G418. DS-*Bam*HI C-Luc is equivalent to *oriP-Bam*HI C-Luc except that it lacks the FR of *oriP*. *oriP*-minus lacks *oriP* and contains a 232-bp insertion at the *Msc*I site within the G418 resistance gene. Competitor lacks *oriP* and contains a 220-bp deletion between the *Bsa*A1 and *Msc*I sites within the G418 resistance gene. Arrows above the derivatives of the G418 resistance genes within each construct indicate the primer binding sites used for the quantitative PCR assay. The sizes of amplified fragments generated for each construct are noted at the right.

negative human osteosarcoma cell line (2). DG75 cells were grown in RPMI 1640 containing 10% calf serum, streptomycin sulfate (0.2 mg/ml), and penicillin G potassium (200 U/ml), and 143B cells were grown in Dulbecco's modified Eagle's medium-high glucose (DMEM-HG) containing 10% calf serum, streptomycin sulfate (0.2 mg/ml), and penicillin G potassium (200 U/ml).

The cell lines used and generated for the short-term replication assays include 143B, 143/EBNA-1 (28), 143/NΔ450-641 (30), 143/NΔ330-641, and 143/NΔ450-618. These 143-derived cell lines that stably express a derivative of EBNA-1 and hygromycin B phosphotransferase (17, 44) conferring resistance to hygromycin were grown in DMEM-HG containing 10% calf serum, streptomycin sulfate (0.2 mg/ml), penicillin G potassium (200 U/ml), and hygromycin (150 μg/ml).

The cell lines generated in this study for use in the long-term replication assays

include several clones of 143/EBNA-1/*oriP-Bam*HI C-Luc and one clone of 143/NΔ330-641/*oriP-Bam*HI C-Luc. The 143/EBNA-1-derived cell lines that also maintain *oriP-Bam*HI C-Luc plasmids were grown as described above except for drug selection. G418 (600 μg/ml) was used instead of hygromycin for selection of clonal cell lines after the introduction of plasmids that confer resistance to G418 via the aminoglycoside phosphotransferase II gene (10) (see below).

**Luciferase assays.** For transient transcription assays where activation of transcription from the FR-TK-Luc reporter was measured in DG75 cells, 0.5 μg of FR-TK-Luc reporter, 2.5 μg effector, and 2.5 μg of vector (5 μg for vector alone) were electroporated per $5 \times 10^6$ cells per 0.5 ml of RPMI–10% calf serum–0.05 M HEPES (pH 7.4). Electroporation was performed by using previously established conditions (using a rise time of 800 ns, a voltage peak of 820 V, and a fall time of 31 ms) (22). When inhibition of activation of transcription was measured, 0.5 μg of FR-TK-Luc reporter and 2.5 μg of each effector (5 μg for vector alone) were electroporated per $5 \times 10^6$ cells as described above. When activation of transcription from the *oriP-Bam*HI C-Luc reporter was measured in DG75 cells, 0.5 μg of *oriP-Bam*HI C-Luc reporter, 2.5 μg of effector, and 2.5 μg of vector (5 μg for vector alone) were electroporated into $5 \times 10^6$ cells as described above. When inhibition of transcription from the *oriP-Bam*HI C-Luc reporter was measured in DG75 cells, 0.5 μg of reporter and 2.5 μg of each effector (5 μg for vector alone) were electroporated into $5 \times 10^6$ cells as described above.

For transient transcription assays where either activation or inhibition of activation of transcription from the FR-TK-Luc reporter was measured in 143B cells, either 0.5 μg of FR-TK-Luc reporter and 2.5 μg of effector or 0.5 μg of FR-TK-Luc reporter and 2.5 μg of effector (5 μg for vector alone) were electroporated into $5 \times 10^6$ cells per 0.5 ml of DMEM-HG–10% calf serum–0.05 M HEPES (pH 7.4). Electroporation was performed by using previously established conditions (using a rise time of 800 ns, a voltage peak of 1,020 V, and a fall time of 25 ms) (22). When activation of transcription from the *oriP-Bam*HI C-Luc reporter was measured in 143B cells, 10 μg of *oriP-Bam*HI C-Luc reporter and 10 μg of effector were electroporated into $5 \times 10^6$ cells as described above. When inhibition of transcription from the *oriP-Bam*HI C-Luc reporter was measured in 143B cells, 5 μg of reporter and 10 μg of effector (15 μg for vector alone) were electroporated into $5 \times 10^6$ cells as described above.

After the introduction of DNA, cells were plated in either RPMI–10% calf serum (DG75 cells) or DMEM-HG–10% calf serum (143B cells). Cells were harvested at 48 h, counted, and lysed at a concentration of $10^5$ cells/μl in reporter lysis buffer, and luciferase assays (6) were performed as instructed by the manufacturer of the luciferase assay system (Promega), using a luminometer (Monolight 2010; Analytical Luminescence Laboratory). The data from the luciferase assays were analyzed by the Wilcoxon rank sum test (19).

**Protein expression.** Expression of each derivative of EBNA-1 was confirmed for the transient transcription and replication assays. The protein expression levels of the cell lines that stably express either wild-type EBNA-1 or various derivatives of EBNA-1 (143/EBNA-1, 143/NΔ330-641, 143/NΔ450-641, and 143/NΔ450-618) used in the short-term replication assays were quantified by a modified quantitative Western blot assay (7). On each gel, 0.3, 1.2, 3, 12, and 30 ng of an affinity-purified, bacterially expressed derivative of EBNA-1, NΔ407-641 (previously referred to as NΔ407) (27), and two dilutions of each cell line ($2 \times 10^4$ to $4 \times 10^5$ cell equivalents) were loaded. (The concentration of NΔ407-641 was measured by comparison with known standards on Coomassie blue-stained gels.) Samples were run on a 10% polyacrylamide gel and transferred to nitrocellulose. The primary antibody used in this analysis was a 1:500 dilution of an affinity-purified polyclonal rabbit, anti-cro-EBNA-1-βgal, which includes residues 7 to 37 and 420 to 617 of EBNA-1 (42). The secondary antibody, $^{35}$S-donkey anti-rabbit immunoglobulin G from Amersham, was used at a concentration of 0.1 μCi/ml in BLOTTO (1× phosphate-buffered saline, 1% nonfat dry milk, 0.05% Tween 20). Blots were quantified with a PhosphorImager (Molecular Dynamics), and protein expression levels were calculated by interpolating results from a graph plotting PhosphorImager units versus nanograms of NΔ407-641.

**Long-term replication assays.** For each cell line tested, 143/EBNA-1 and 143/NΔ330-641, 10 μg of *oriP-Bam*HI C-Luc DNA was electroporated into an aliquot of $10^7$ cells as described above. After 48 h, cells were plated at $10^2$, $10^3$, $10^4$, $10^5$, and $10^6$ cells per 150- by 25-mm plate, and selection with 600 μg of G418 per ml was introduced. One to eight drug-resistant clones of each cell type were picked from the plates after 14 to 21 days and expanded for further analysis (20). The average number of copies of *oriP-Bam*HI C-Luc DNA maintained in these clones was determined by the quantitative PCR assay (see below) or by Southern blotting (21, 40). The remaining colonies on the plates were fixed with 3.7% formaldehyde for 10 min, stained for 5 min with 0.14% methylene blue in ethanol, and counted to determine the efficiency of colony formation (21).

**Quantitative PCR assay. (i) Introduction of DNA into 143 cells.** A total of $2 \times 10^7$ 143B cells, or derived cell lines, were resuspended in 1 ml of DMEM-HG–10% calf serum–50 mM HEPES (pH 7.4 to 7.6). Ten micrograms of each DNA (*oriP-Bam*HI C-Luc or DS-*Bam*HI C-Luc, *oriP*-minus, and a derivative of EBNA-1 or vector) was electroporated into $10^7$ 143 cells/0.5 ml of DMEM-HG–10% calf serum–0.05 M HEPES (pH 7.4) as described above. One electroporated sample was plated per 150- by 25-mm plate in 20 ml of DMEM-HG–10% calf serum for 94 to 98 h. Samples were then harvested by the Hirt extraction procedure (18). The supernatant was transferred to a fresh tube and incubated for 2 h at 50°C with RNase A (100 μg/ml, final concentration) and subsequently incubated for 2 h at 45°C with proteinase K (200 μg/ml, final concentration). Samples were extracted with phenol, chloroform, and ether. DNA was ethanol precipitated, and samples were resuspended in 1× TE (10 mM Tris-HCl, 1 mM EDTA [pH 7.4]) and subsequently digested with *Bam*HI to linearize the plasmid DNA and with *Dpn*I to digest any nonmethylated, or unreplicated, DNA. Samples were extracted with phenol-chloroform and chloroform. DNA was ethanol precipitated, and samples were resuspended in 1× TE to $10^5$ cell equivalents/μl.

**(ii) Generation of competitor DNA.** Twenty micrograms of competitor DNA was linearized with *Bam*HI. Samples were extracted once each with an equal volume of phenol-chloroform and an equal volume of chloroform. Five molar ammonium acetate was added to give a final concentration of 0.3 M, and DNA was precipitated with the addition of 2.5 volumes of 100% ethanol. Competitor DNA was resuspended in 1× TE, and its concentration was determined with a Hoescht dye assay (24).

**(iii) PCR assay.** The following assay is a modification of the quantitative PCR assays described by Piatak et al. (31) and Casella (7). Five PCRs were performed per sample, using increasing amounts of competitor DNA per reaction (five of the six following amounts per sample: 0.025 pg [corresponding to approximately $4.9 \times 10^3$ molecules of competitor DNA], 0.10 pg [$2.0 \times 10^4$ molecules], 0.40 pg [$7.9 \times 10^4$ molecules], 1.6 pg [$3.2 \times 10^5$ molecules], 6.4 pg [$1.3 \times 10^6$ molecules], and 26 pg [$5.0 \times 10^6$ molecules]); 1 μl of sample DNA ($10^5$ cell equivalents) and 89 μl of reaction mix were added to each tube and mixed (giving a final amount of 0.2 mM each deoxynucleoside triphosphate, 0.2 μM each primer, 0.05 to 0.1 μCi of [$^{32}$P]dCTP, and 2.5 U of *Taq* polymerase [Boehringer] per PCR). The 100-μl total reaction volume was overlaid with 70 μl of mineral oil in 500-μl GeneAmp tubes (Perkin-Elmer). DNA was amplified by using a Thermocycler 480 (Perkin-Elmer) under the following conditions: 94°C for 5 min; 94°C for 30 s, 55°C for 30 s, and 72°C for 1 or 2 min, 25 to 30 cycles; 72°C for 10 min; and 4°C hold. Upon completion of the PCR, the mineral oil was removed by extraction with 2 volumes of chloroform. Amplified products were then transferred to a screw-cap tube, ethanol precipitated as described above to remove the majority of the unincorporated [$^{32}$P]dCTP, and resuspended in 100 μl of TE. Then 15 μl of the PCR mix from each sample was loaded on a 1.5% agarose gel, and the gel was electrophoresed in 1× TBE (0.9 M Tris-borate, 1 mM EDTA) overnight at 40 V. The gel was then fixed in 10% ethanol–10% acetic acid for 30 min and dried, and data were analyzed with a PhosphorImager (Molecular Dynamics).

The primers used in the PCRs included 5′CGCTTAACAGCGTCAACAGC GTGCC3′, which lies in the herpes simplex virus (HSV) thymidine kinase (TK) promoter region, and 5′ACGATTCCGAAGCCCAACCTTTCA3′, which lies in the 3′ nontranscribed region of the G418 resistance gene. The sizes of the amplified products generated by using these primers are 742 bp for competitor DNA, 964 bp for *oriP-Bam*HI C-Luc DNA or DS-*Bam*HI C-Luc DNA, and 1,197 bp for *oriP*-minus DNA.

**(iv) Data analysis.** The primers used in the quantitative PCR assay allow the simultaneous amplification of the competitor DNA, the sample DNA, and the *oriP*-minus DNA. When the number of templates of competitor DNA is equal to the number of templates of sample DNA, the two are amplified with equal efficiency.

For data analysis, log (molecules of competitor) versus log [PhosphorImager units of competitor/(PhosphorImager units of *oriP-Bam*HI C-Luc × 1/1.31)] was plotted, and the number of *Dpn*I-resistant molecules per $10^5$ cell equivalents of sample was determined from the inverse log of the intercept. That is, when the counts of competitor equals counts of *oriP-Bam*HI C-Luc corrected for the number of dCTPs in the amplified template, the log of 1/1 equals 0. {The ratios of dCTPs per double-stranded template used to correct for signal by incorporation of [$^{32}$P]dCTP is 435/435, or 1, for competitor DNA [435 dCTPs per double-stranded template], 569/435, or 1.31, for sample DNA [*oriP-Bam*HI C-Luc or DS-*Bam*HI C-Luc, 569 dCTPs per double-stranded template], and 677/435, or 1.56, for *oriP*-minus DNA [677 dCTPs per double-stranded template]. To ensure that the *Dpn*I digestions had gone to completion, the graph log (molecules competitor) versus log [counts of competitor/(counts of *oriP*-minus × 1/1.56)] was also plotted and analyzed accordingly.} Therefore, the inverse log of the intercept equals the number of *Dpn*I-resistant molecules in the sample per $10^5$ cell equivalents, assuming 100% transfection efficiency. The data presented in this report were corrected for the actual transfection efficiency of each cell line used (see below). The data from the replication experiments were analyzed by the Wilcoxon rank sum test (19).

**(v) Validation of the quantitative PCR assay.** The ability of the quantitative PCR assay to measure accurately the level of *Dpn*I-resistant DNA in the samples tested was validated by independent measurements of identical samples of *Dpn*I-resistant DNA by Southern analysis. The level of replication measured by the quantitative PCR assay was within approximately twofold of the level measured by Southern analysis for both EBNA-1-positive cell lines that stably maintained plasmids containing *oriP* and 143B cells that transiently expressed NΔ330-641 and replicated *oriP-Bam*HI C-Luc (data not shown). The quantitative PCR assay was also validated by comparing known amounts of CsCl-purified *oriP-Bam*HI C-Luc DNA or *oriP*-minus DNA against known amounts of CsCl-purified competitor DNA in the presence of $10^5$ cell equivalents of Hirt extracts from 143B cells. The amount of DNAs present as measured by quantitative PCR was consistent with the concentration of DNA present in the original stocks as measured by Hoescht dye assay. This measurement indicates that all templates used in the assay are amplified with similar efficiencies under the conditions

tested (data not shown). The data conformed to theoretical requirements for linearity (34).

**Transfection efficiency of 143-derived cell lines.** To determine the transfection efficiency of each cell line used in the short-term replication analysis, 20 μg of plasmid CMV-βgal (formerly known as EQ176; contains the β-galactosidase [β-Gal] gene driven by the CMV promoter [39]) was electroporated into $10^7$ 143B cells or derivative cell lines per 0.5 ml of DMEM-HG–10% calf serum–0.05 M HEPES (pH 7.4) under the electroporation conditions described above. Cells were plated in DMEM-HG–10% calf serum for 24 h. At that time, cells were counted and replated at $10^5$ and $5 \times 10^5$ cells per 60-mm-diameter gridded dish. At 48 h, cells were fixed with 2 ml of 1% glutaraldehyde–100 mM $NaPO_4$ (pH 7.0)–1 mM $MgCl_2$ for 15 min. Cells were rinsed with phosphate-buffered saline and incubated in 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) cleavage buffer [10 mM $NaPO_4$ (pH 7.0), 150 mM $MgCl_2$, 3.3 mM $K_4Fe(CN)_6 3H_2O$, 3.3 mM $K_3Fe(CN)_6$, 0.2% X-Gal] for 24 h to detect β-Gal expression (25). All blue and white cells were counted in each of 10 squares for each replicate of each sample, and the results for the β-Gal assay (percent blue cells) are reported as the transfection efficiency of each cell line.

The transfection efficiencies (means ± standard deviations) for the cell lines used in the replication studies using quantitative PCR are as follows: 143B cells, 15 ± 5.6%, $n = 3$; 143/EBNA-1 cells, 39 ± 9.0%, $n = 3$; 143/NΔ330-641 cells, 9.6 ± 1.9%, $n = 3$; 143/NΔ450-641 cells, 20 ± 5.2%, $n = 4$; 143/NΔ450-618 cells, 7.6 ± 1.4%, $n = 4$; and DG75 cells, 7.1 ± 5.7%, $n = 8$. Transfection efficiencies were also remeasured and reconfirmed throughout the course of the replication experiments.

## RESULTS

**Experimental approach.** We screened derivatives of EBNA-1 for the ability to inhibit functions of wild-type EBNA-1. Because these derivatives obviously might retain some of wild-type EBNA-1's functions, they were first tested for the intrinsic ability to support transcription and replication. The derivatives of EBNA-1 were tested in short-term assays in which vectors expressing the derivatives (effectors) were introduced into cells along with reporter DNAs and the resulting transcription or replication was measured 48 or 96 h later. They were also tested in short- or long-term replication experiments in which the derivatives of EBNA-1 were expressed constitutively in the recipient cells such that the level of the expression of the derivative was known. Appropriate reporter DNAs were introduced into these recipient cells, and the resulting replication was measured both at 96 h and 4 or more weeks later. The derivatives were assayed for their inhibition of wild-type functions in parallel assays.

**Activation of transcription of heterologous and native EBV promoters by derivatives of EBNA-1.** Derivatives of EBNA-1 (Fig. 1A) were tested for the ability to activate transcription from either a heterologous promoter, the HSV TK promoter, or a native EBV promoter, the *Bam*HI C promoter (Fig. 1B), by measuring luciferase activity at 48 h following transfection of both DG75 cells and 143B cells (Table 1). NΔ450-641 and NΔ450-618, which lacked all three linking domains of EBNA-1 but maintained the ability to bind to DNA, did not activate transcription relative to vector alone ($P = 0.24$). These derivatives activated neither the HSV TK promoter juxtaposed to the FR nor the *Bam*HI C promoter in its natural context downstream of *oriP* in either DG75 or 143B cells (Table 1). A related derivative of EBNA-1 with a different NLS and an epitope tag was previously reported to activate transcription from FR-TK-CAT in a third cell line (1). Our findings indicate that the activation domain identified within this derivative is not sufficient to mediate activation of transcription of nearby promoters under the conditions tested. A third mutant that we analyzed, NΔ330-641, which can link DNAs with specific binding sites for EBNA-1 in vitro (27) and contains two of the three linking domains of EBNA-1, activates transcription inefficiently from the *Bam*HI C promoter via *oriP* in both DG75 and 143B cells ($P = 0.018$) relative to vector alone (Table 1). This weak ability of NΔ330-641 to activate transcription from the less complex FR-TK promoter was not observed (see Discussion).

TABLE 1. Activation of transcription by EBNA-1 and three of its derivatives

| Effector | Relative fold transactivation[a] | | | |
|---|---|---|---|---|
| | DG75 cells | | 143B cells | |
| | FR-TK-Luc | *oriP-Bam*HI C-Luc | FR-TK-Luc | *oriP-Bam*HI C-Luc |
| Vector | 1 | 1[b] | 1 | 1 |
| EBNA-1 | 28 ± 9.9[c] | 17 ± 18[b] | 11 ± 2.4 | 56 ± 13 |
| NΔ330-641 | 2.6 ± 0.87 | 4.7 ± 2.7 | 1.4 ± 0.35 | 5.1 ± 2.7 |
| NΔ450-641 | 2.5 ± 1.7 | 2.5 ± 2.0 | 2.1 ± 1.6 | 2.5 ± 2.4 |
| NΔ450-618 | 3.6 ± 4.2 | 1.8 ± 1.4 | 1.3 ± 0.34 | 2.1 ± 1.7 |

[a] Four electroporations were pooled in pairs and plated in either RPMI–10% calf serum or DMEM-HG–10% calf serum for 48 h for DG75 or 143B cells, respectively, prior to performance of luciferase assays. The average of the pairs of these samples represents one experiment. Data, measured as relative light units of luciferase activity, are expressed as fold transactivation relative to that of vector alone, which has been set to 1 (= 10,143 ± 8,254, 3,555 ± 1,279, 1,931 ± 895, and 5,351 ± 199 relative light units for FR-TK-Luc and *oriP-Bam*HI C-Luc in DG75 cells and FR-TK-Luc and *oriP-Bam*HI C-Luc in 143B cells, respectively). Data presented represent averages of three experiments ± standard deviations unless otherwise indicated.

[b] Data from part of the experiment included in Table 2 for *oriP-Bam*HI C-Luc in DG75 cells. See Materials and Methods.

[c] Average ± standard deviation of two experiments.

**Derivatives of EBNA-1 inhibit transcription by EBNA-1.** To determine whether derivatives of EBNA-1 could inhibit transcription activated by wild-type EBNA-1, we introduced together plasmids expressing wild-type EBNA-1, a derivative of EBNA-1, and either *oriP-Bam*HI C-Luc or FR-TK-Luc into 143B or DG75 cells (Table 2). Two derivatives of EBNA-1, NΔ450-641 and NΔ450-618, that could not activate transcription (Table 1) were efficient inhibitors of transcription by wild-type EBNA-1. NΔ450-641 could inhibit wild-type EBNA-1's ability to activate transcription from the *oriP-Bam*HI C-Luc reporter by approximately 81 and 84% in DG75 and 143B cells, respectively, and the FR-TK-Luc reporter by approximately 97 and 86% in DG75 and 143B cells, respectively. NΔ450-618 was also able to inhibit transcription from the *oriP-Bam*HI C-Luc

TABLE 2. Derivatives of EBNA-1 function as dominant-negative inhibitors of transcription by EBNA-1

| Effector | Relative fold transactivation[a] | | | |
|---|---|---|---|---|
| | DG75 cells | | 143B cells | |
| | FR-TK-Luc | *oriP-Bam*HI C-Luc | FR-TK-Luc | *oriP-Bam*HI C-Luc |
| Vector | 1 | 1[b] | 1 | 1 |
| EBNA-1, Vector | 41 ± 25 | 17 ± 18[b] | 16 ± 8.3 | 31 ± 20 |
| EBNA-1, NΔ330-641 | 2.9 ± 1.2 | 17 ± 12 | 3.4 ± 1.6 | 18 ± 12 |
| EBNA-1, NΔ450-641 | 1.1 ± 0.60 | 3.2 ± 1.3 | 2.3 ± 0.67 | 4.9 ± 1.5 |
| EBNA-1, NΔ450-618 | 0.94 ± 0.17 | 2.4 ± 0.65 | 1.1 ± 0.46 | 2.9 ± 1.2 |

[a] Four electroporations were pooled in pairs and plated in either RPMI–10% calf serum or DMEM-HG–10% calf serum for 48 h for DG75 or 143B cells, respectively, prior to performance of luciferase assays. The average of the pairs of these samples represents one experiment. Data, measured as relative light units of luciferase activity, are expressed as fold transactivation relative to vector alone, which has been set to 1 (= 10,748 ± 7,571, 3,555 ± 1,279, 1,892 ± 791, and 1,268 ± 803 relative light units for FR-TK-Luc and *oriP-Bam*HI C-Luc in DG75 cells and FR-TK-Luc and *oriP-Bam*HI C-Luc in 143B cells, respectively). Data presented represent averages of three experiments ± standard deviations.

[b] Data from part of the experiment included in Table 1 for *oriP-Bam*HI C-Luc in DG75 cells. See Materials and Methods.

reporter by approximately 96 and 91% in DG75 and 143B cells, respectively, and from the FR-TK-Luc reporter by approximately 98 and 93% in DG75 and 143B cells, respectively. NΔ330-641, which did not activate FR-TK-Luc but did activate *oriP-Bam*HI C-Luc inefficiently, did inhibit wild-type EBNA-1's activation of FR-TK-Luc but not that of *oriP-Bam*HI C-Luc (Table 2). This promoter-dependent, dominant-negative phenotype of NΔ330-641 may reflect its residual transcriptional activation functions. The *Bam*HI C promoter is more complex than the FR-TK promoter and is enhanced by *oriP* (14), which can be linked by EBNA-1 (43). NΔ330-641, which contains two of the three linking domains of EBNA-1 (unlike NΔ450-641 and NΔ450-618), may associate with wild-type EBNA-1 and with cellular factors that bind to the *Bam*HI C promoter but not the FR-TK promoter, thereby allowing transcriptional activation to occur.

To gauge the level of transient expression of the inhibitors of EBNA-1 relative to that of wild-type EBNA-1, quantitative Western blot assays were performed. Lysates of DG75 cells transfected with 10 μg of effector DNA encoding either wild-type EBNA-1 or one of the derivatives of EBNA-1, NΔ330-641, NΔ450-641, or NΔ450-618, were analyzed for levels of protein expression at 48 h postelectroporation. The levels of expression of the derivatives were determined by interpolating from a standard curve of purified, bacterially expressed NΔ407-641 (27) included on each blot. The level of protein expressed for each derivative is given as an average ± standard deviation relative to the value for wild-type EBNA-1, which is set to 1. The levels of expression are as follows: for NΔ330-641, 0.84 ± 0.083, $n = 4$; for NΔ450-641, 9.5 ± 7.8, $n = 4$; and for NΔ450-618, 0.36 ± 0.13, $n = 2$. Thus, derivatives of EBNA-1 that are transiently expressed between approximately 40% as efficiently as wild-type EBNA-1 to approximately 10-fold more efficiently than wild-type EBNA-1 can function as dominant-negative inhibitors. Importantly, their levels of inhibition do not merely reflect their level of expression.

**Short-term replication of plasmids containing *oriP* by derivatives of EBNA-1.** Assays for replication were performed in cell lines engineered to express EBNA-1 and its derivatives. Quantitative Western blot assays were performed in triplicate for clonal derivatives of 143B cells that stably express either wild-type EBNA-1 or one of the derivatives of EBNA-1, NΔ330-641, NΔ450-641, or NΔ450-618, to analyze levels of protein expression (Fig. 2 and data not shown). The level of protein expressed for each cell line is given as an average of three experiments ± standard deviation relative to the value for wild-type EBNA-1 in 143/EBNA-1 cells, which is set to 1. The levels of expression are as follows: for 143/NΔ330-641, 1.4 ± 0.65; for 143/NΔ450-641, 21 ± 0.53; and for 143/NΔ450-618, 1.5 ± 0.51. Thus, derivatives of EBNA-1 are expressed as efficiently as or more efficiently than wild-type EBNA-1 in each cell line.

The abilities of derivatives of EBNA-1 to support replication of an *oriP*-containing plasmid were then tested in 143B cells and the 143 cell lines that stably express derivatives of EBNA-1. At 96 h posttransfection, *oriP-Bam*HI C-Luc was efficiently replicated in 143/wild-type EBNA-1 cells, whereas replicated *oriP-Bam*HI C-Luc was not detected in 143B cells that lacked EBNA-1 (Fig. 3; Tables 3 and 4). The *oriP*-positive plasmid replicated in 143/NΔ330-641 cells to approximately 4% of the level to which it replicated in 143/EBNA-1 cells that stably express similar levels of wild-type EBNA-1 (Table 3). In contrast, the internal control plasmid that lacked *oriP*, *oriP*-minus, did not replicate detectably. Neither the *oriP*-positive plasmid nor the *oriP*-negative control plasmid replicated in 143/NΔ450-641 and 143/NΔ450-618 cells (Table 3). Although a low level of *Dpn*I-resistant signal from *oriP-Bam*HI C-Luc was occasionally detected in 143/NΔ450-641 cells, this signal was less than the lowest concentration of competitor DNA used, or less than 1% of the *Dpn*I-resistant signal from *oriP-Bam*HI C-Luc detected in 143/EBNA-1 cells.

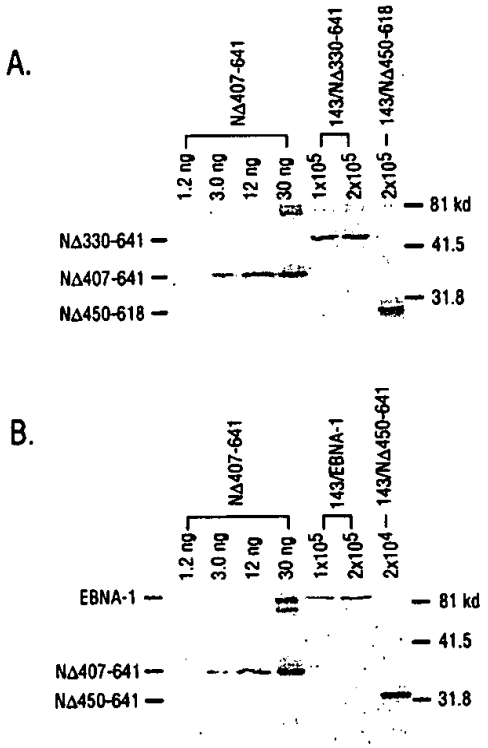


FIG. 2. Stable expression of derivatives of EBNA-1 in 143B cells. (A) Quantitative Western blot of 143/NΔ330-641 and 143/NΔ450-618 cells. (B) Quantitative Western blot of 143/EBNA-1 and 143/NΔ450-641 cells. Clonal cell lines were generated as described in Materials and Methods, and quantitative Western blot assays were performed in triplicate for each cell line to determine protein expression levels. A 1:500 dilution of affinity-purified rabbit anti-EBNA-1 which recognizes primarily the carboxy-terminal third of EBNA-1 was used as the primary antibody. The secondary antibody was a 0.1-μCi/ml dilution of $^{35}$S-labeled donkey anti-rabbit immunoglobulin G. Blots were analyzed with a PhosphorImager (Molecular Dynamics), and protein expression levels were determined by interpolating from a standard curve of purified, bacterially expressed NΔ407-641 (27) included on each blot. Positions of molecular weight standards are noted at the right, migration of proteins is shown at the left, and the amount of NΔ407-641 or the number of cell equivalents of lysate is noted above each lane.

A transfection assay was used to provide an independent approach to study replication mediated by derivatives of EBNA-1. Vectors that express each derivative were introduced along with *oriP-Bam*HI C-Luc and *oriP*-minus, and replication was measured in both 143B cells and DG75 cells. By 96 h postelectroporation, the *oriP*-containing plasmid had replicated efficiently in the presence of wild-type EBNA-1, whereas replication was not detected in 143B cells that lacked EBNA-1 (Fig. 3A; Table 4). The derivatives of EBNA-1, NΔ450-641 and NΔ450-618, which lack all three linking domains but can bind to DNA, could not support replication of either *oriP-Bam*HI C-Luc or *oriP*-minus detectably when expressed transiently in 143B cells ($P = 0.5$, relative to vector). The derivative NΔ330-641 which contains two of the three linking domains of EBNA-1 did support replication of the *oriP*-containing plasmid with approximately 11% of the efficiency of wild-type EBNA-1 in 143B cells ($P = 0.05$, relative to vector) and approximately

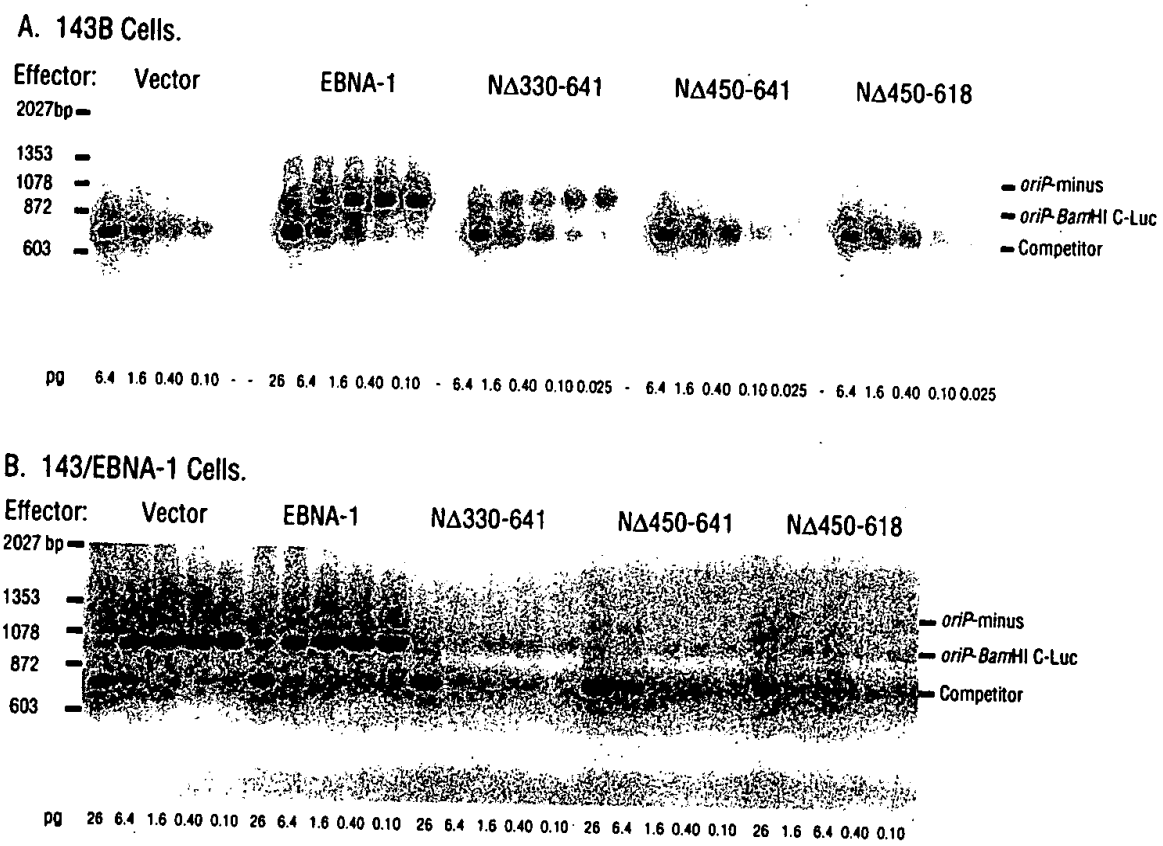


FIG. 3. Short-term replication of plasmids containing *oriP* as determined by quantitative PCR. (A) Short-term replication in 143B cells that transiently express derivatives of EBNA-1. Ten micrograms each of *oriP-Bam*HI C-Luc, *oriP*-minus DNA, and the indicated vector or effector DNA was introduced by electroporation into $10^7$ 143B cells that transiently express the indicated derivatives of EBNA-1. (B) Inhibition of short-term replication of plasmids containing *oriP* by derivatives of EBNA-1 in 143 cells that stably express wild-type EBNA-1. Ten micrograms each of *oriP-Bam*HI C-Luc, *oriP*-minus DNA, and the indicated vector or effector DNA was introduced by electroporation into $10^7$ 143 cells that stably express wild-type EBNA-1. Low-molecular-weight DNA was isolated at 94 to 98 h; plasmid DNA was purified by Hirt extraction (18), digested with *Bam*HI and *Dpn*I, and analyzed by quantitative PCR. Reaction mixtures were separated by electrophoresis on 1.5% agarose gels, data were collected with a PhosphorImager (Molecular Dynamics), and the amount of replicated DNA was determined as described in Materials and Methods. The amount of competitor DNA introduced into the PCR is indicated below each lane. No sample was loaded in the lanes labeled with dashes. Standards for DNA molecular weights are noted at the left, and locations of *oriP*-minus, *oriP-Bam*HI C-Luc, and competitor DNA are noted at the right.

23% of the efficiency of wild-type EBNA-1 in DG75 cells (Table 4).

To test if the replication measured in the presence of NΔ330-641 has the hallmarks of bona fide *oriP*-dependent replication, we studied replication of a replication-defective (38) plasmid that contains the dyad symmetry element but lacks the FR of *oriP*, DS-*Bam*HI C-Luc. If the requirements for replication in *cis* mediated by NΔ330-641 were similar to those of wild-type EBNA-1, we would predict that neither wild-type EBNA-1 nor NΔ330-641 would support replication of this mutant plasmid. Analysis of DS-*Bam*HI C-Luc introduced into 143B cells confirmed that neither wild-type EBNA-1 nor NΔ330-641 support replication of this plasmid detectably (unpublished data).

**Limited replication of plasmids containing *oriP* by derivatives of EBNA-1.** To determine what fraction of the *oriP*-containing plasmids that are replicated by 96 h in cells expressing EBNA-1 are also maintained over time as plasmids, we generated G418-resistant cell lines that stably maintain *oriP-Bam*HI C-Luc. We compared the level of *oriP*-dependent replication detected at 96 h in the presence of wild-type EBNA-1 to the number of replicated *oriP-Bam*HI C-Luc molecules maintained in a series of clonal 143/EBNA-1/*oriP-Bam*HI C-Luc cell lines. In one experiment, replication was measured at 96 h postelectroporation from a single transfection of *oriP-Bam*HI C-Luc into 143/EBNA-1 cells. This transfection was performed in parallel with a single transfection of *oriP-Bam*HI C-Luc into 143/EBNA-1 cells that was used to generate eight clonal 143/EBNA-1/*oriP-Bam*HI C-Luc cell lines. In this experiment, approximately 17 copies of *oriP-Bam*HI C-Luc were detected per transfected 143/EBNA-1 cell at 96 h ($n = 1$), and 41% of this level of *oriP-Bam*HI C-Luc was maintained as plasmids in clonal 143/EBNA-1/*oriP-Bam*HI C-Luc cell lines ($n = 8$) (Table 5). When these data are combined with measurements from three additional transfection experiments of *oriP-Bam*HI C-Luc into 143/EBNA-1 cells (Table 3) and two other transfection experiments used to generate independent clonal 143/EBNA-1/*oriP-Bam*HI C-Luc cell lines, we found similar results. Approximately 22 ± 7.9 replicated copies of *oriP-Bam*HI C-Luc per cell were detected at 96 h ($n = 4$), and 50% of this level was measured on average in 13 independent clonal 143/EBNA-1/*oriP-Bam*HI C-Luc cell lines after 4 to 6 weeks of drug selection (Table 5). These results indicate that in the presence of wild-type EBNA-1, the majority of the *oriP*-containing plasmids that are replicated initially in cells are maintained as plasmids in those cells that survive selection.

To determine whether NΔ330-641 supports the replication of *oriP*-containing plasmids during prolonged propagation of the host cells, we attempted to generate clonal cell lines of 143/NΔ330-641 that maintained *oriP-Bam*HI C-Luc. The effi-

TABLE 3. NΔ330-641 supports replication of plasmids containing *oriP* with approximately 4% of the efficiency of wild-type EBNA-1 in 143B cells selected to express wild-type EBNA-1 or derivatives of EBNA-1

| Cell Line | Relative efficiency of replication[a] | |
|---|---|---|
| | *oriP-Bam*HI C-Luc | *oriP*-minus |
| 143B | <1.4[b] | <1.4[b] |
| 143/EBNA-1 | 100 | <2.1[b] |
| 143/NΔ330-641 | 3.9 ± 2.0 | <2.1[b] |
| 143/NΔ450-641 | <1.0[b] | <1.0[b] |
| 143/NΔ450-618 | <2.7[b] | <2.7[b] |

[a] Ten micrograms of each DNA was introduced into $10^7$ cells of each cell line by electroporation. Low-molecular-weight DNA was isolated at 94 to 98 h, and the relative amount of *Dpn*I-resistant plasmids was determined by quantitative PCR and was corrected for the transfection efficiency of each cell line as described in Materials and Methods. The relative efficiency of replication by each derivative of EBNA-1 is expressed as a percentage of *Dpn*I-resistant DNA relative to that replicated by wild-type EBNA-1, which is set to 100% (= 24 ± 8.5 copies of *Dpn*I-resistant DNA per transfected cell). Data represent averages of three experiments ± standard deviation (σ).

[b] The level of *Dpn*I-resistant DNA was less than the lowest amount used for the competitor DNA and is therefore reported as less than an average of the lowest amount per transfected cell that could be determined by interpolation from the competitor DNA curve.

ciency of generating G418-resistant colonies in 143/NΔ330-641 cells was less than 0.4% of the efficiency of generating G418-resistant colonies in 143/EBNA-1 cells, after correction for the difference in transfection efficiencies of the two cell lines (data not shown; see Materials and Methods). One G418-resistant 143/NΔ330-641/*oriP-Bam*HI C-Luc cell line was generated and was found to contain approximately one integrated copy of *oriP-Bam*HI C-Luc, as measured by quantitative PCR and Southern analysis (Table 5 and data not shown). Taken together, these results indicate that although NΔ330-641 can inefficiently replicate *oriP*-containing plasmids in short-term assays, NΔ330-641 is not capable of maintaining *oriP*-containing plasmids in proliferating host cells.

TABLE 4. NΔ330-641 supports replication of plasmids containing *oriP* with 11 and 23% of the efficiency of wild-type EBNA-1 in 143B and DG75 cells, respectively, at 96 h postelectroporation[a]

| Effector | Relative efficiency of replication in: | | | |
|---|---|---|---|---|
| | 143B cells | | DG75 cells | |
| | *oriP-Bam*HI C-Luc | *oriP*-minus | *oriP-Bam*HI C-Luc | *oriP*-minus |
| Vector | <2.0 ± 1.0[b] | <2.0 ± 1.0[b] | 6.9 ± 0.58 | 5.5 ± 0.12 |
| EBNA-1 | 100 | <2.8[b] | 100 | 4.5 ± 0.27 |
| NΔ330-641 | 11 ± 8.0 | <2.0 ± 1.0[b] | 28 ± 19 | 3.6 ± 1.8 |
| NΔ450-641 | <2.0 ± 1.0[b] | <2.0 ± 1.0[b] | — | — |
| NΔ450-618 | <2.0 ± 1.0[b] | <2.0 ± 1.0[b] | — | — |

[a] Ten micrograms of each DNA was introduced into $10^7$ 143B or DG75 cells by electroporation. Low-molecular-weight DNA was isolated at 94 to 98 h, and the relative amount of *Dpn*I-resistant plasmids was determined by quantitative PCR and was corrected for transfection efficiency each cell line as described in Materials and Methods. The relative efficiency of replication by each derivative of EBNA-1 is expressed as a percentage of *Dpn*I-resistant DNA relative to that replicated by wild-type EBNA-1, which is set to 100% (= 50 ± 30 and 13 ± 0.50 copies of *Dpn*I-resistant DNA per transfected cell for 143B and DG75 cells, respectively). Data for 143B cells represent averages of three experiments ± standard deviation (σ), one of which is shown in Fig. 3A. Data for DG75 cells represent an averages of two experiments ± standard deviation (σ). Residual *Dpn*I resistant, *oriP*-minus DNA was detected in Hirt extracts from DG75 cells in some experiments. Therefore, the efficiency of replication of *oriP-Bam*HI C-Luc by NΔ330-641 is calculated as (28 − 3.6)/(100 − 4.5) = 23% relative to wild-type EBNA-1. —, not tested.

[b] The level of *Dpn*I-resistant DNA was less than the lowest amount used for the competitor DNA and is therefore reported as less than an average of the lowest amount per transfected cell that could be determined by interpolation from the competitor DNA curve.

TABLE 5. Replication of *oriP-Bam*HI C-Luc by wild-type EBNA-1 or NΔ330-641 at 94 to 98 h and at 4 to 6 weeks

| Expt | Cells | No. of replicated *oriP-Bam*HI C-Luc plasmids/cell[a] | | No. of samples |
|---|---|---|---|---|
| | | 94–98 h | 4–6 wk | |
| 1 | 143/EBNA-1 | 17[b] | —[c] | 1 |
| | 143/EBNA-1 | — | 6.9 ± 3.8[d] | 8 |
| 2 | 143/EBNA-1 | 22 ± 7.8[e] | — | 4 |
| | 143/EBNA-1 | — | 11 ± 7.8[f] | 13 |
| | 143/NΔ330-641 | 0.94 ± 0.48[g] | — | 3 |
| | 143/NΔ330-641 | — | 0[h] | 1 |

[a] Ten micrograms of each DNA was introduced into $10^7$ 143/EBNA-1 or 143/NΔ330-641 cells by electroporation. Low-molecular-weight DNA was isolated at 94 to 98 h for 143/EBNA-1 and 143/NΔ330-641 samples or at 4 to 6 weeks after the generation of G418-resistant clonal cell lines for 143/EBNA-1/*oriP-Bam*HI C-Luc samples. The copy number of plasmids per cell was determined by quantitative PCR or Southern blotting as described in Materials and Methods and is expressed as an average ± standard deviation (σ).

[b] Datum represents a single transfection, corrected for transfection efficiency, performed in parallel with two pooled transfections used to generate clonal 143/EBNA-1 cell lines stably maintaining the *oriP-Bam*HI C-Luc plasmid described in experiment 1.

[c] —, not tested.

[d] Average of eight clones of 143/EBNA-1 cell lines stably maintaining *oriP-Bam*HI C-Luc.

[e] Average of the three experiments from Table 4 and the 143/EBNA-1 94- to 98-h replication sample from experiment 1, corrected for transfection efficiency of 143/EBNA-1 cells.

[f] Average of 13 independent G418-resistant clonal cell lines from three assays ($n$ = 8 from experiment 1, $n$ = 2 from the second assay, and $n$ = 3 from the third assay).

[g] Average of the three experiments from Table 3, corrected for transfection efficiency of 143/NΔ330-641 cells.

[h] One million cell equivalents of total cellular extracts of one G418-resistant clonal 143/NΔ330-641 cell line were analyzed by Southern blotting. *oriP-Bam*HI C-Luc had integrated into chromosomal DNA.

**Dominant-negative inhibition of replication of plasmids containing *oriP* by derivatives of EBNA-1.** To determine whether derivatives of EBNA-1 could inhibit wild type EBNA-1-dependent replication of plasmids containing *oriP*, we introduced the plasmids expressing derivatives of EBNA-1, *oriP-Bam*HI C-Luc, and *oriP*-minus into 143/EBNA-1 cells (Fig. 3B; Table 6). We found that NΔ450-618 and NΔ450-641, which could not support plasmid replication, were able to inhibit wild-type EBNA-1's ability to replicate *oriP-Bam*HI C-Luc by approximately 95 and 98%, respectively. Similar levels of inhibition occurred in a second host cell: NΔ450-641 inhibited replication of *oriP* supported by transiently introduced wild-type EBNA-1 by 94% in DG75 cells (Table 6). NΔ330-641, which could support plasmid replication, could also function less effectively as an inhibitor of EBNA-1, was able to inhibit replication by approximately 76% in 143/EBNA-1 cells. These results indicate that derivatives of EBNA-1 which inhibit transcription can also inhibit replication by wild-type EBNA-1.

**Mechanism of action of dominant-negative inhibition by derivatives of EBNA-1.** We examined whether a derivative of EBNA-1 that does not bind to DNA inhibits wild-type EBNA-1's functions efficiently. To this end, we designed a derivative, NΔ450-458,483-641 (Fig. 1A), based on previously described derivatives of EBNA-1 that can dimerize but cannot bind to DNA (8). NΔ450-458,483-641 is predicted to form homodimers as well as heterodimers with derivatives of EBNA-1 that contain a wild-type DNA binding and dimerization do-

TABLE 6. Derivatives of EBNA-1 can inhibit transient replication of plasmids containing *oriP* in 143B cells selected to express wild-type EBNA-1 and in DG75 cells transiently expressing wild-type EBNA-1

| Effector | Relative efficiency of replication[a] in: | | | |
|---|---|---|---|---|
| | 143/EBNA-1 cells | | DG75 cells | |
| | *oriP-Bam*HI C-Luc | *oriP*-minus | *oriP-Bam*HI C-Luc | *oriP*-minus |
| Vector | 100 | <1.3[b] | 100[c] | 2.4 ± 0.59[c] |
| EBNA-1 | 95 ± 28 | <1.3[b] | — | — |
| NΔ330-641 | 24 ± 16[c] | <0.97[b] | — | — |
| NΔ450-641 | 1.6 ± 0.64 | <0.82 ± 0.49[b] | 5.6 ± 0.44[c] | 1.3 ± 0.21[c] |
| NΔ450-618 | 4.5 ± 2.6 | <0.97 ± 0.46[b] | — | — |

[a] Ten micrograms of *oriP-Bam*HI C-Luc, *oriP*-minus, and effector DNA (plus 10 μg of EBNA-1 DNA for DG75 cells) was introduced into $10^7$ cells of 143/EBNA-1 and DG75 cells by electroporation. Low-molecular-weight DNA was isolated at 94 to 98 h, and the relative amount of *Dpn*I-resistant plasmids was determined by quantitative PCR and was corrected for the transfection efficiency of each cell line as described in the Materials and Methods. The relative efficiency of replication by each derivative of EBNA-1 is expressed as a percentage of *Dpn*I-resistant DNA relative to that replicated by wild-type EBNA-1, which is set to 100% (= 39 ± 19 and 34 ± 1.4 copies of *Dpn*I-resistant DNA per transfected 143/EBNA-1 and DG75 cell, respectively). Unless indicated otherwise, data represent averages of three experiments ± standard deviations (σ), one of which is shown in Fig. 3B. —, not tested.

[b] The level of *Dpn*I-resistant DNA was less than the lowest amount used for the competitor DNA in three experiments and is therefore reported as less than an average of the lowest amount per transfected cell that could be determined by interpolation from the competitor DNA curve. Low levels of *Dpn*I-resistant *oriP*-minus DNA were detected in low-molecular-weight DNA from DG75 cells, as indicated in column 5 of table.

[c] Average of two experiments ± standard deviation (σ).

main (8). However, NΔ450-458,483-641 is predicted to lack the ability to bind to DNA either as a homodimer or as a heterodimer because it lacks critical residues required for contacting DNA (5, 8). NΔ450-458,483-641 inhibited transcription of FR-TK-Luc by wild-type EBNA-1 in 143B cells by approximately 50%, whereas NΔ450-641, which can dimerize and bind to DNA, inhibited transcription by 96% (Table 7). Thus, the data indicate that although heterodimerization may play a role in the ability of derivatives of EBNA-1 to function as dominant-negative inhibitors, these inhibitors of EBNA-1 must bind to DNA to function efficiently.

## DISCUSSION

We have identified dominant-negative mutants of EBNA-1. These derivatives have been assayed both for their residual levels of wild-type functions and for the ability to inhibit the functions of wild-type EBNA-1. Two derivatives of EBNA-1 tested, NΔ450-641 and NΔ450-618, did not activate transcription detectably under the conditions tested. The carboxy-terminal acidic domain present in NΔ450-641 does not activate transcription from either the FR-TK-Luc or the *oriP-Bam*HI C-Luc reporters in either 143B or DG75 cells. One derivative of EBNA-1, NΔ330-641, activates transcription from a native EBV promoter with approximately 9% of the efficiency of wild-type EBNA-1 in 143B cells ($P = 0.018$) and approximately 28% of the efficiency of wild-type EBNA-1 in DG75 cells ($P = 0.018$). NΔ330-641 does not activate the FR-TK-Luc reporter construct in either 143B cells or DG75 cells, probably because the FR-TK promoter lacks elements found in the EBV *Bam*HI C promoter critical for NΔ330-641's residual activity.

The derivatives of EBNA-1 that fail to activate transcription also fail to support detectable replication in short-term assays (Tables 3 and 4). NΔ330-641, which activates limited transcription from the *Bam*HI C promoter, also supports replication of *oriP* inefficiently in short-term but not long-term assays (Tables 3 to 5). These findings underscore the parallel between EBNA-1's contribution to transcription and replication. The derivatives of EBNA-1 which lack residual levels of wild-type function but bind DNA site specifically can act as efficient inhibitors of wild-type EBNA-1 (Tables 2 and 6). NΔ330-641 inefficiently inhibits wild-type EBNA-1 in these assays, in which it retains detectable levels of wild-type function probably because it can contribute positively to these assays (Tables 2 and 6). As NΔ330-641 does not activate the FR-TK promoter, it efficiently inhibits wild-type EBNA-1's activation of this synthetic promoter. NΔ330-641's phenotype of both activation and inhibition has been observed for the E2 repressor proteins of papillomaviruses. E2TR of human papillomavirus type 18 (11) and E2TR and E8/E2TR of bovine papillomavirus type 1 (35, 41), in addition to repressing transcription, can activate transcription weakly from both derivatives of homologous as well as heterologous promoters, depending on the promoter context studied.

There are several possible ways that these derivatives of EBNA-1 could function as dominant-negative inhibitors of EBNA-1. First, they could form homodimers, bind to DNA, and prevent wild-type EBNA-1 from binding and activating transcription. Second, they could form heterodimers with wild-type EBNA-1 and inhibit homodimers of wild-type EBNA-1 from binding to DNA. Heterodimerization may also reduce the amount of homodimers of wild-type EBNA-1 in the cell available to bind to DNA (see below). Alternatively, these derivatives could form heterodimers with wild-type EBNA-1, bind to DNA as heterodimers, but prevent the wild-type EBNA-1 moiety from functioning. It is likely that a combination of these modes of inhibition occurs within the cell.

It appears that derivatives of EBNA-1 do need to bind DNA specifically in order to inhibit wild-type EBNA-1 efficiently. NΔ450-458,483-641 lacks residues required to bind to DNA but maintains residues required for dimerization. Similar derivatives of EBNA-1 have been found previously to heterodimerize with derivatives of EBNA-1 that contain wild-type DNA binding and dimerization domains and thereby prevent binding to DNA in vitro (8). Therefore, although NΔ450-458,483-641 may form either homodimers or heterodimers with wild-type EBNA-1, these complexes may not bind to DNA. NΔ450-458,483-641 inhibits transcription by wild-type EBNA-1 by only 50%. Thus, although the ability of dominant-negative mutants to heterodimerize may play a role in their inhibition of transcription, their binding to DNA, either as a

TABLE 7. A derivative of EBNA-1 that does not bind to DNA is an inefficient inhibitor of transcription by wild-type EBNA-1 in 143B cells

| Effector | Relative fold transactivation of FR-TK-Luc[a] |
|---|---|
| Vector | 1 |
| EBNA-1, vector | 87 ± 49 |
| EBNA-1, NΔ450-641 | 3.7 ± 1.8 |
| EBNA-1, NΔ450-458,483-641 | 44 ± 23 |

[a] Four electroporations were pooled in pairs and plated in DMEM-HG–10% calf serum for 48 h prior to performance of luciferase assays. The average of the pairs of these samples represents one experiment. Data, measured as relative light units of luciferase activity, are expressed as fold transactivation relative to that of vector alone, which has been set to 1 (= 1,858 ± 106 relative light units). Data represents averages ± standard deviations of three experiments.

homodimer or as a heterodimer with wild-type EBNA-1, is required for them to act efficiently.

Our findings also indicate that the dominant-negative mutants of EBNA-1 are likely to inhibit wild-type EBNA-1 when they occupy only a subset of the binding sites on *oriP*. Although 20 binding sites for EBNA-1 are present in the FR of *oriP*, approximately six to seven binding sites are sufficient to function as an enhancer of transcription in the presence of EBNA-1 (9, 46). Similarly, a minimal FR of seven binding sites in the presence of the dyad symmetry element of *oriP* is sufficient for replication (46). In the experiments in which vectors for EBNA-1 and its dominant-negative mutants were introduced simultaneously (Table 2), it is unlikely that the derivatives were able to occupy all 20 binding sites within the FR of *oriP* as either homodimers or as heterodimers with wild-type EBNA-1, because the proteins are expressed at similar levels. Instead, it is likely that a few inhibitors bound randomly throughout the FR can disrupt a higher-order structure that is formed by multiple wild-type EBNA-1 dimers bound to DNA.

NΔ330-641 activates replication of a plasmid that contains *oriP* with approximately 4 to 23% of the efficiency of wild-type EBNA-1 in 143B-derived and DG75 cell lines by 96 h post-electroporation. However, NΔ330-641 is not capable of supporting detectable replication of that plasmid in proliferating cells after long times; wild-type EBNA-1 supports both replication and maintenance of *oriP* replicons in proliferating cells. NΔ330-641, like wild-type EBNA-1, cannot support replication of a plasmid that contains a mutant origin lacking the family of repeats of *oriP* in 143B cells. Thus, although NΔ330-641 can replicate DNA in an *oriP*-dependent manner, it cannot maintain that replicated DNA in cells over time. One simple interpretation of this finding is that the two linking domains of EBNA-1 retained in NΔ330-641 are sufficient to support its residual function but the third linking domain and any other activities in the deleted region are required for wild-type function. The defect in replication mediated by NΔ330-641 could be explained by its supporting initiation of replication 50% as frequently as does wild-type EBNA-1. The approximately four cellular S phases that occur in the 96 h between electroporation and assaying for replication would yield a signal of NΔ330-641 equal to $0.5^4$, or 6% of wild-type EBNA-1—the signal detected.

Alternatively, NΔ330-641 may support initiation of replication as efficiently as wild-type EBNA-1 but be one-half as efficient as wild-type EBNA-1 at retaining that DNA in the nucleus after mitosis (facilitating segregation to daughter cells), which would yield the signals detected at 96 h. A specific DNA retention function has been attributed to wild-type EBNA-1, which has been used to identify replication-competent human DNA sequences (23). Assays that measure the number of *oriP* plasmids in a single cell and, subsequently, in each of its progeny have not yet been developed. When they are, they will provide an unambiguous way of examining the mechanism of the defect in NΔ330-641.

We are now in a position to use these dominant-negative inhibitors of EBNA-1 to investigate EBNA-1's role both early and late in infection and immortalization of primary B lymphocytes by EBV. As the findings in Table 6 indicate, when a recipient cell is expressing wild-type EBNA-1 before expressing a newly introduced dominant-negative mutant, the mutant still inhibits replication by wild-type EBNA-1 efficiently. It will be particularly intriguing to determine if the continued function of EBNA-1 is required for the continued proliferation of normal B cells and neoplastic B cells infected by EBV.

## ACKNOWLEDGMENTS

We thank Timothy Middleton for being the catalyst for the use of a quantitative PCR assay and Todd Hopkins and Amy Sarkis for technical assistance. We also thank Norman Drinkwater, David Mackey, and Mary Ellen Perry for critical review of the manuscript.

This research was supported by Public Health Service grants CA-22443, CA-07175, and T32-CA-09135.

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